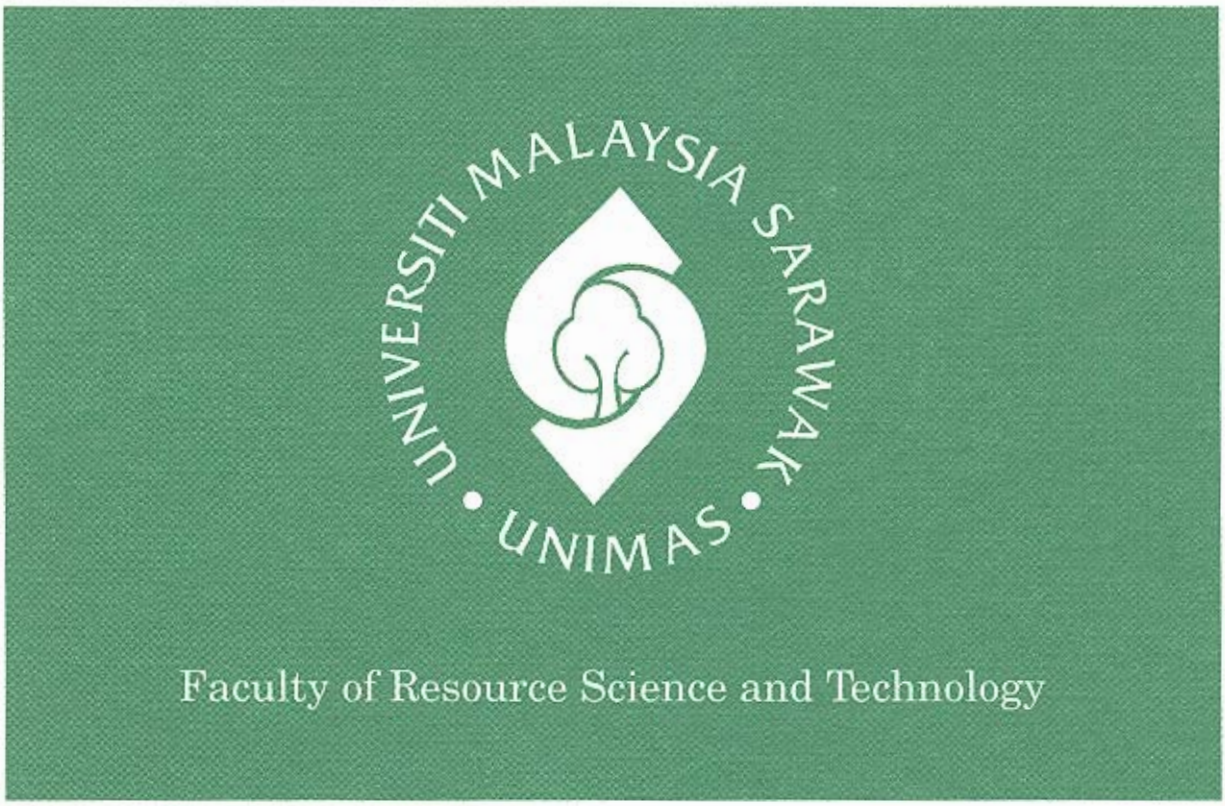

APPLICATION OF PULSED - FIELD GEL ELECTROPHORESIS IN STUDYING DIVERSITY OF *Esherichai Coli* FROM WASTE WATER AND RIVER WATER

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Bachelor of Science with Honours
(Resource Biotechnology)
2005

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This project is submitted in partial fulfilment of the requirements of the degree of
Bachelor of Science with Honours
(Resource Biotechnology)

FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY
UNIVERSITI MALAYSIA SARAWAK
2005

ACKNOWLEDGEMENT

First of all, I would like to thank my supervisor, Assoc. Prof. Dr. Kasing Apun, for her guidance and helps towards completion of this project and my special gratitude to my family, for their support and love.

My special appreciation to Miss Chong Yee Ling for her advice and assistance in this project, Encik Azis dan Encik Amin for their technical support and all the post graduate students in the Microbiology Lab, who had helped me a lot in improving my laboratory skills.

Not to forget, my fellow partner in the lab, Miss Aini Hayati and Miss Nor Fazila for their companionship and also to my coursemate, Miss Heng Wei Sze for her support and encouragement.

Finally, I would like to thank all the lecturers in Faculty of Resource Science and Technology for providing me knowledge during all these years of undergraduate study.

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ABSTRACT

Pulsed-field gel electrophoresis (PFGE) was used to compare and analyzed 42 isolates of *Escherichia coli* from five different water sources namely Kuap River, Melaban River, Jernang River, Sarawak General Hospital, and Matang Landfill. Sample collection was done on monthly basis (September, October, and November, 2004). PFGE profile generated by *Xba*I as restriction endonuclease produced 40 restriction endonuclease digestion profiles (REDPs) with 5 to 16 DNA fragments while one isolates being untypable. The PFGE profiles were then analyzed using RAPDistance package to determine the distance matrix relationship (D value) among the *E. coli* strains. The results showed that high diversity among *E. coli* strains isolated with highest D value of 1.00 and lowest D value of 0.25. This indicates that the *E. coli* strains were not closely related even though samples were taken from the same location and varied between different months.

Key words: *Escherichia coli*, PFGE, *Xba*I, RAPDistance

ABSTRAK

Dalam kajian ini, 'pulsed-field gel electrophoresis' (PFGE) telah digunakan untuk membandingkan dan menganalisa 42 pencilan Escherichia coli dari lima sumber air yang berlainan iaitu Sungai Kuap, Sungai Melaban, Sungai Jernang, Hospital Umum Sarawak, dan pusat pelupusan sampah Matang. Pengumpulan sampel dijalankan berdasarkan bulan (September, Oktober, dan November, 2004). Profil PFGE yang dijana menggunakan Xbal sebagai enzim pemotong menghasilkan 40 profil penghazaman enzim pemotong dengan 5 hingga 16 jalur DNA dan satu pencilan tidak dapat diprofilkan. Profil PFGE kemudiannya dianalisa menggunakan pakej RAPDistance untuk mengenalpasti jarak perhubungan (nilai D) antara jujukan E. coli. Keputusan menunjukkan kadar kepelbagaian jujukan E. coli yang tinggi iaitu nilai D tertinggi adalah 1.00 dan nilai D terendah adalah 0.25. Ini menunjukkan bahawa E. coli mempunyai perkaitan yang jauh walaupun dari tempat yang sama dan berbeza mengikut bulan.

Kata kunci: Escherichia coli, PFGE, XbaI, RAPDistance

1.0 INTRODUCTION

1.1 Background

Water is the dominant compound on earth which occupies nearly three-fourths of the earth surface. It is the most essential resource for all living organisms and also provides a habitat for several of microorganisms. Sulfur bacteria, iron bacteria, free-living spiral forms, certain pigmented and non-pigmented species, and some spore formers are some of the microorganisms that can be found in natural waters. Pathogenic microbes do not usually make up a part of the normal flora in any area. Therefore, contamination form external sources such as sewage make the water become dangerous.

Water source for community in Sarawak is supplied from the river water. There is always a possibility that there might be a contamination to the water system that comes from chemical or biological source, which can cause disease epidemics. There are only minor cases of water contamination that relates to chemical contaminant such as lead, copper, fluoride, or nitrate if compared to microbial infection, such as *Campylobacter jejuni* (Hänninen *et al.*, 2003), *Shigella sonnei* (Nester *et al.*, 2001), and *Escherichia coli* (Bitton, 1994). In Malaysia, water from irrigation ditches, ponds, swamps and river water is used to cultivate crops such as vegetables. These sources of water might contain bacterial pathogen that can cause disease. Consumption of these contaminated crops could result in the transmission of pathogenic microorganisms to consumers (Thong *et al.*, 2002). Recent study by Apun *et al.* (2003) only emphasized on investigation of raw beef meat in Sabah and Sarawak infected with *Escherichia coli*. Therefore,

sources of fecal contamination in our waterways must be identified in order to adequately address water quality problems and protect public health.

Furthermore, various infectious diseases such as typhoid fever, cholera, and hepatitis A can be transmitted in water. Since it is impossible to test water for all pathogens, certain organisms are used as an indicator that is called coliform bacteria. Among the well known coliform bacteria is *Escherichia coli* which is found in the intestines of virtually all human. The term coliform is used to describe all Gram-negative non-spore-forming, facultative organisms that ferment lactose and produce acid and gas. Their presence in water is a strong indication of recent sewage or waste contamination. Compared to other fecal coliform such as *Enterobacter aerogenes, E. coli* is considered a more specific indicator of fecal coliform, as other fecal coliforms have been found in ambient waters in the absence of apparent fecal pollution and may establish viable populations when high level of carbohydrates are available as nutrient source (Toranzos *et al.*, 1997). Furthermore, its weak ability to replicate in natural environments features the discriminatory power of *E. coli* as an indicator organism (Byamukama *et al.*, 2000).

Epidemiological studies of pathogen are very crucial in order to control spreading of dangerous pathogens. Conventional epidemiologic typing methods based on phenotypic characterization of organisms such as serotyping, biotyping, and antibiograms have many limitations. Therefore, strain typing of bacterial pathogens have been introduced which allow better distinctness among bacterial and fungal pathogen. Strain typing is essential to identify the clonality of strains involved either in local epidemics or global epidemiology (Thong *et al.*, 2002). Furthermore, it is also extremely important in recognizing outbreaks of infection,

detecting the cross-transmission of pathogens, determining the source of infection, and recognizing particularly virulent strains of organisms (Olive and Bean, 1999). Strain typing can be done by using a number of different approaches, such as pulsed-field gel electrophoresis (PFGE), ribotyping, random amplified polymorphic DNA (RAPD), DNA fingerprinting using the mobile genetic element IS*200* and amplified fragment length polymorphism (AFLP).

Pulsed-field gel electrophoresis (PFGE) is a technique that is capable in providing a chromosomal restriction profile typically composed of 5 to 20 distinct, well resolved fragments ranging form 10 to 800 kb (Tenover *et al.*, 1995). All these fragment can be determined as a pattern of distinct bands by PFGE by using a specially designed chamber that locate the agarose gel between three sets of electrode that form a hexagon around the gel. A system has been proposed by Tenover *et al.* (1995) for standardizing the interpretation of PFGE patterns in relation to determining strain relatedness. Bacterial isolates that yield the same PFGE patterns are considered as the same strain. A closely related strain has a difference of one to three bands. Isolates differing by four to six bands, representing two independent genetic changes, are possibly related. Bacterial isolates containing six or more band differences, representatives of three or more genetic changes are considered unrelated.

In this study, a Dice Coefficient will be used to quantify the similarity between PFGE banding pattern. The use of the Dice Coefficient is to measure genetic similarity assumes that bands of identical size are, with high probability, genetically homologous (Davis *et al.*, 2003). *Xba*I will be used in this study as restriction endonuclease. It has been reported that *Xba*I provide

the best discrimination and cheaper, with the most easily interpreted restriction fragments for studies of characterization of *E. coli* (Święcicka *et al.*, 2003; Thong *et al.*, 2002).

1.2 Statement of Problem

Escherichia coli has been used for more than hundred years for routine monitoring of the microbiological safety of drinking water. Using an indicator to assess water quality is useful because indicator organisms are easier to detect compare to actual pathogens (Toranzos and McFeters, 1997). Therefore, establishment of data collection of *E. coli* strains from waste water and river water is important for comparing the *E. coli* strain. From there, any difference between the *E. coli* strains collected from waste water and river water can be obtained and be determined whether there are distinct pattern or similar pattern between *E. coli* from waste water and clean water. Previous study by Liew (2002), shows that there is genetic variation of *E. coli* isolates from the same geographical area that are Sungai Sebayor and Sungai Kuap.

Furthermore, existence of marker gene through the DNA pattern can be identified as an assumption that *E. coli* from waste water can undergo adaptation to suit its environment. In additions, occurrence of pathogenic *E. coli* in our waste water system can be discovered through this study. From there, further step can be done to avoid water-borne disease outbreak such as treat the water using chlorine, ultra-violet light, or ozone, all of which act to kill or inactivate *E. coli*.

1.3 Objective

This research is undertaken with the following objectives;

1. To analyze the diversity of *E. coli* strain from waste water and river water by using pulsed-field gel electrophoresis.
2. Determination of genetic relatedness between *E. coli* from different source and locations in Sarawak.
3. To determine if there is variation of *E. coli* strain between month and whether it is correlated with the physical characteristics such as pH and temperature.

2.0 LITERATURE REVIEW

2.1 *Escherichia coli*

The normal flora is important in protecting the body against the invasion by pathogens. *E. coli* is the predominant facultative anaerobic bacterium in the human colonic flora which is usually remains harmlessly confined to the intestinal lumen. This bacteria normally colonizes the infant gastrointestinal tract within hours of life. However, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even *E. coli* strains of normal flora can cause infection such as gastrointestinal diseases. *E. coli* has been largely derived from strains recovered from humans, zoo, or domestic animals (Cobbold and Desmarchelier, 2001; Izumikawa *et al.*, 1998; Krause *et al.*, 1996; and Tamura *et al.*, 1996), and environments contaminated with human and animal waste (McLellan *et al.*, 2001; Parveen *et al.*, 1997; and Rosas *et al.*, 1997).

The pathogenic strain of *E. coli* includes enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and enterohemorrhagic *E. coli* (EHEC). Strains in these groups own virulence factors that allow them to be intestinal pathogens compared to other strains of *E. coli* (Bitton, 1994).

2.2 Pathogenic strain of *Escherichia coli*

Enterotoxigenic *Escherichia coli* or ETEC is an *E. coli* strains that elaborate at least one member of two defined group of enterotoxins: ST and LT. These strains can cause diarrhea

through the action of the enterotoxins LT and ST. These strains may express an LT only, an ST only, or both. ETEC colonize the surface of the small bowel mucosa secrete their enterotoxins, giving rise to a net secretory state (Hirayama, 1995; Hirst, 1995; Hol *et al.*, 1995; O'Brien *et al.*, 1996, Sears and Kaper, 1996). Infection of ETEC will cause two major clinical syndromes; weanling diarrhea among children in the developing world, and traveler's diarrhea. Most infants in areas of endemic infection get infected with ETEC upon weaning and among adult it occur when adult travelers from developed world visiting areas where ETEC infection is endemic (Nataro and Kaper, 1998).

The epidemiologic pattern of ETEC disease can be recognized by several factors; (i) mucosal immunity to ETEC infection develops in exposed individuals, (ii) immune asymptomatic individuals may shed large numbers of virulent ETEC organisms in the stool, and (iii) the infection need a relatively high infection dose. ETEC infection usually occurs through contaminated water and food. In addition, it also tends to infect during warm, wet months, when multiplication of ETEC in water and food is the most efficient (Arduino and DuPont, 1993).

Enteropathogenic *Escherichia coli* (EPEC) which is a fecal-oral transmitted has been connected to infant diarrhea in the developing world and primarily infect infants younger than 2 years. Studies have showed that EPEC infection is based on physiological basis for resistance rather than host immunity or exposure to EPEC. Even though EPEC usually infect infants, several cases of outbreaks have been reported to infect adults. Studies have show that EPEC infection occur due to ingestion of a large inoculum from a common source and the adults that were infected had health problems such as diabetics, those with achlorhydria and the elderly. In

addition, EPEC can also be transmitted thorough contaminated hands and contaminated weaning foods or formula.

Compared to other pathogenic *E. coli* strains, enterohemorrhagic *Escherichia coli* (EHEC) can be recognized through the effect of its infections. There are two key epidemiologic observations of EHEC. The first one is hemorrhagic colitis (HC) that is characterized by severe crampy abdominal pain, watery diarrhea followed by grossly bloody diarrhea, and a little or no fever. This illness is associated with consumption of undercooked hamburger at a fast-food restaurant chain (Nataro and Kaper, 1998).

The second observation is the association of sporadic cases of hemolytic uremic syndrome (HUS) with fecal cytotoxin and cytotoxin-producing *E. coli* in stools. HUS which is defined by the triad of acute renal failure, thrombocytopenia and microangiopathic hemolytic anemia was already known to be preceded typically by a bloody diarrhea illness indistinguishable from HC (Pickering *et al.*, 1994; Griffin, 1995). Stool cultures from patients that are infected with these strains yielded a rarely isolated *E. coli* serotype O157:H7 which produce toxin known as Shiga-like toxin (Stx). This potent cytotoxin is the factor that leads to death and many other symptoms in patients infected with EHEC. The Stx family contain two major member, immunologically non-cross-reactive groups called Stx1 and Stx2. Stx1 is identical to Shiga toxin from *Shigella dysentriae* 1 (Khan *et al.*, 2002).

EHEC can be transmitted by food and water and from person-to-person. Consumption of contaminated food, especially beef and contamination of water sources, including recreational

water, well water, and even a municipal water systems have also been associated with outbreaks of EHEC. The outbreak happened due to the municipal water system, which affected 243 individuals and caused four deaths, resulted from an improperly repaired water system which allowed unchlorinated water to be widely distributed (Swerdlow *et al.*, 1992).

Enteroaggregative *Escherichia coli* is a new category of diarrhogenic *E. coli* to describe strain expressing aggregative adherence (AA). It is currently defined as *E. coli* strain that do not secretes enterotoxins LT or ST and adhere to cells in an AA pattern. Studies have showed that EAEC is associated with developing populations. The symptoms of infection are watery, mucoid, secretory diarrheal illness with low-grade fever and little to no vomiting. Four outbreaks of EAEC diarrhea in UK that involve 19, 10, 51, and 53 patients respectively have been reported in 1994. Each of the outbreaks was associated with consumption of a restaurant meal, but no single source could be implicated (Smith *et al.*, 1994).

Another pathogenic *E. coli* strain is Enteroinvasive *Escherichia co*li (EIEC). Infection of EIEC present most commonly as watery diarrhea, which can be indistinguishable from the secretory diarrhea seen with ETEC. However, severe illness has also been reported which is dysentery syndrome, manifested as blood, mucus and leucocytes in the stool, and fever (Nataro and Kaper, 1998).

2.3 Strain Typing

Strain typing is an important part of epidemiological investigation of pathogen infections. Strain typing data have been helpful in investigating outbreaks caused by wide range of bacterial pathogens, including methicilin-resistant strains of *Staphylococcus aureus* (MRSA) (El-Ahami *et al.*, 1991), vancomycin-resistant enterococci (VRE) (Montecalvo *et al.*, 1994), *Pseudomonas aeruginosa* (Sader *et al.*, 1993), and *Klebsiella pneumoniae* (Gouby *et al.*, 1994). Typing methods can be divided into two categories, namely phenotypic methods and genotypic methods (Tenover *et al.*, 1997). Phenotypic methods distinguished the products of gene expression in order to differentiate strains. Phenotypic properties that can be identified including biochemical profiles, bacteriophage types, antigens present on the cell's surface, and antimicrobial susceptibility profiles. These properties vary based on changes in growth conditions, growth phase, and spontaneous mutation. Whereas in genotypic methods, they are depend on analysis of the genetic structure of an organism and the presence of extrachromosomal DNA. Furthermore, it also include polymorphism in DNA restriction patterns based on cleavage of the chromosome by enzymes that cut the DNA into hundreds of fragments that is called frequent cutters or into infrequent cutters that cleave the DNA into 10 to 30 fragments. All typing system can be characterized in terms of typeability, reproduceability, discriminatory power, ease of performance, and ease of interpretation (Arbeit, 1995).

2.4 Pulsed-Field Gel Electrophoresis

Strain typing such as pulsed-field gel electrophoresis (PFGE) that fall in genotypic method category can be used to detect genomic variations that characterized certain strain. Any differences that exist in the DNA sequences can be used to distinguish among strains that are phenotypically identical. This is very important in tracing epidemics of foodborne or waterborne illness. Typing based on the fingerprinting pattern of bacterial genomic DNA after PFGE has been reported to be convenient tool for epidemiological studies of bacterial infection (Gordillo *et al.*, 1993). Moreover, PFGE is different from conventional electrophoresis in the way that current is applied. In PFGE, the current is applied first in one direction from one set of electrodes, then transfer to the second set of the electrodes for a short period of time known as 'pulse', and then transfer to the third set of the electrodes. Thus, the electric field that causes the DNA to migrate in the gel is provided in pulses that alternate from three sets of electrodes. This causes the DNA to wiggle through the gel, and the back-and-forth movement results in a higher level of fragment resolution seen with the technique (Tenover *et al.*, 1997).

Analysis of bacterial DNA using PFGE has at least three significant uses. First, a physical map of the bacterial chromosome can be developed by using known genes as probes (Smith *et al.*, 1987). As the result, the size of bacterial genome can be determined directly and libraries of defined subregions of the chromosome can be constructed by electroeluting the large restriction fragments from the agarose gel. Second, transposon insertion into previously uncloned loci can be mapped (Murakami *et al.*, 1989). Lastly, PFGE is known to be highly effective in molecular epidemiologic studies of bacterial isolates and, in particular, is superior to both phenotypic and

Southern blotting techniques in discriminating among isolates of *E. coli, staphylococcus aureus*, and other species (Arbeit *et al.*, 1990).

The purpose of PFGE is to provide improved resolution of large DNA molecules compared to conventional agarose gel electrophoresis (Towner and Cockayne, 1993). The gels and buffers that are used are same and the main difference is in the way that the electrical field is applied. There are different types of PFGE; UPFGE (unidirectional pulse-field gel electrophoresis), OFAGE (orthogonal field alternation gel electrophoresis), FIGE (field inversion gel electrophoresis), TAFE (transverse alternating field electrophoresis), CHEF (contour-clamped homogenous electric fields), PACE (programmable, autonomously-controlled electrode system), and RGE (rotating gel electrophoresis) (Towner and Cockayne, 1993). The major differences among the various systems are; 1) whether straight line can be obtained; 2) the speed of separation; 3) the resolution within a particular size range; and 4) how large a portion of the gel provides useful separation (Birren and Lai, 1993). However, the most popular pulse-field system is CHEF technique. The CHEF's system depends on a hexagonal electrode array to produce highly uniform electric field. The overall effect is to produce straight lanes and good resolution of DNA fragments (Towner and Cockayne, 1993).

From previous studies, PFGE has proven to be superior to most other method for molecular typing. It is highly discriminatory and superior to most method for analysis of *E. coli*, vancomycin-resistant enterococci, *S. aureus*, *Acinetobacter* sp., *Pseudomonas aeruginosa*, and *M. avium* (Arbeit *et al.*, 1990; Murray *et al.*, 1990; Poh *et al.*, 1992; Arbeit *et al.*, 1993; Saulnier *et al.*, 1993; Schlichting *et al.*, 1993; Tenover *et al.*, 1994; Grundmann *et al.*, 1995; Barbier *et al.*,

1996; Liu *et al.*,1997). PFGE was more discriminatory than repetitive element sequence-based PCR (Rep-PCR) for differentiating strains of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex, vancomycin-resistant enterococci, *Neisseria gonorrhoeae*, and *P. aeruginosa*. (Grundmann *et al.*, 1995; Barbier *et al.*, 1996; Poh *et al.*, 1996; Liu *et al.*, 1997;).

It is also more discriminatory than phage typing since it could further differentiate the four phage type of *Serovar Typhi* into 21 PFGE profiles (Thong *et al.*, 2002). PFGE was also shown to be very useful in delineating the genetic variability of the *S. enterica* strain and was a crucial epidemiological tool, as the DNA fingerprints generated were stable and reproducible and all the strains were typeable (Thong *et al.*, 2002). In epidemiological studies of *E. coli*, PFGE has been proven to be a very useful tool because of its power of discrimination (Izumiya *et al.*, 1997; Welinder-Olsson *et al.*, 2002).

3.0 MATERIALS AND METHODS

3.1 Sampling Sites and Sample Collection

Samples from river water around Kota Samarahan (Sungai Melaban, Sungai Jernang, and Sungai Kuap) and waste water (Sarawak General Hospital, Kuching and Matang Landfill, Matang) were collected using sterile bottle with five replicates from river water and seven replicates from waste water. Physical test based on pH and temperatures were done on the collected samples using pH meter. Sampling was done on the month of August, September, and October 2004.

3.2 Microbiological Quality Analysis

The microbial water quality analysis was done using Total Plate Count based on the technique in microbiology manual lab (UNIMAS) and total coliform count for coliform bacteria. In Total Plate Count and Total Coliform Count, serial dilution for river water was conducted in 10^{-2} and 10^{-3} whereas for waste water the dilution was 10^{0} and 10^{-1}. Dilution was done using phosphate buffered saline (PBS) pH 7.04. Diluted samples were spread on total plate agar to determine the total count of bacteria and EMB agar for coliform count.

In total plate count, the colonies were counted manually after incubation of 24 hours at 37°C whereas for coliform count, green metallic sheen colonies that were formed on the Petri dish were counted by assuming that one colony was derived from one cell. The colonies were counted manually after incubation period of 24 hours at 37°C.

3.3 Bacterial Isolation and Identification

Green metallic sheen colonies from microbial quality analysis or from enrichment culture in Luria Bertani broth were streak on Eosine Methylene Blue (EMB) Agar (Oxoid, England) by using a loop for bacterial isolation. Agar plates were incubated overnight at 37°C. The next day, bacterial colonies that show green-metallic sheen were subcultured onto Eosine Methylene Blue Agar (Oxoid, England) for confirmation and were incubated overnight at 37°C. Eosine Methylene Blue Agar is a selective medium in which it will exhibit a green metallic sheen by reflected light and dark purple centers by transmitted light for *E. coli* strains.

3.4 Bacterial Growth

The confirmed *E. coli* isolated were grown on Nutrient Agar (Oxoid, England) as working culture and on Tripticase Soy Agar as stock culture.

Gram staining was conducted to examine the cell morphology of the suspected *E. coli* strains using the working culture. More identification steps were done through several standards biochemical test such as Simmon citrate utilization test, methyl red test, *Voges-Proskauer* test, indole test, motility test, SIM test and Kligler Iron Agar (KIA) test (Benson, 1994).

3.5 Preparation of DNA for PFGE

Protocol by Thong *et al.*, 1994 that have been modified was used in this study and completed in four days of work for an overall process.

Single bacterial colony was inoculated from EMB to Tripticase Soy Agar (Becton Dickinson, USA) and subsequently to 10 ml of Brain Heart Infusion broth (Oxoid, England), grow overnight at 37°C with gentle agitation at 120 rpm (Innova 4000, Incubator Shaker Scientific). 1 ml of overnight culture was harvested by centrifugation (Mikro 22R, Hettich Zentrifugen) at 8,000 rpm for 5 minute at 4°C. Cell pellet that was obtained was washed by resuspending it with 1 ml of cold SB (10 mM Tris-base [pH 7.5], 1 M NaCl). Supernatant was discarded and the cell pellet was resuspended in 0.5 ml warm SB. The cell suspension was then transferred into a bijoux and placed in 42°C water bath (Labtech, Daihan). Then, an equal volume of molten 1.5% low-melting point agarose (Ultra Pure, GIBCOBRL USA) diluted in LMP buffer (10 mM Tris-base [pH 7.5], 100 mM EDTA [pH 8.0], and 20 mM NaCl) and was added to the cell suspension. Next, the mixture was pipetted into a plug mould (Bio-Rad Laboratories) and allowed to set at 4°C for 15 minutes. The solidified agarose plugs was placed in 2 ml of lysis solution (10 mM Tris-base [pH 7.5], 50 mM NaCl, 100 mM EDTA [pH 8.0], 0.5% Sarcosyl, 0.5% Brij-58, and 1 mg/ml lysozyme). The DNA plugs were incubated overnight at 37°C in waterbath shaker (Labtech, Daihan).

The next day, the DNA plugs were transferred to new universal bottles containing 2 ml of ES (0.5 M EDTA [pH 8.0], 1% Sarkosyl) with 1 mg/ml Proteinase K. The plugs were incubated

overnight at 50°C in waterbath (Labtech, Daihan) with gentle shaking. After that, the plugs were washed with 2 ml of TE (10 mM Tris-base [pH 8.0], 0.1 mM EDTA [pH 8.0]) for five times at room temperature with gentle agitation at 90 rpm. All transferring of plugs were done with sterile spatula (alcohol-flame). The prepared DNA plugs were stored in 0.5 M EDTA at 4°C or can be used for restriction endonucleases (RE) digestion instantly.

3.6 Negative Control and Positive Control

Untreated DNA samples were ran on the pulsed-field gel with the same electrophoretic conditions for digested DNA as the negative control. This was done to check the purity of genomic DNA embedded in agarose and the chromosomal DNA quality was confirmed by checking whether DNA degradation occurred during storage or any mechanical shearing involved during preparation. For the positive control, wild strain of *E. coli* was used.

3.7 Restriction Endonuclease Digestion

DNA plugs were cut into size of 1 mm x 1 mm x 5 mm using an alcohol-flame scalpel blade and immersed in 1 ml of TE buffer in a 1.5 ml microcentrifuge tube. The plugs were incubated on ice for 1 hour with the tube being inverted occasionally to ensure equilibration. TE was removed and fresh TE was added for further one hour, changing the TE buffer at 30 minutes interval.

Next, the plugs were chilled on ice with 100 µl of 1X restriction endonuclease buffer mix (Buffer D, MBI Fermentas) for 30 minutes. RE buffer will be removed and replaced with a fresh mixture

of 20 units of *Xba*I (MBI Fermentas). The plugs were placed on ice for 20 minutes before incubation at 37°C overnight.

3.8 Running Pulsed-Field Gel Electrophoresis

After the digestion, the slice was equilibrated with 1 ml of 0.5X TBE buffer (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA [pH 8.0]) on ice for 30 minutes. The slice was loaded into the well of 1.2% Ultra Pure PF certified agarose (Bio-Rad Laboratories) prepared in 0.5X TBE buffer. The wells were sealed with the remainder of the molten 1.2% agarose gel. Before performing electrophoresis, the gel was pre-chilled at 4°C for 5 minutes.

PFGE of inserts was performed by using contour-clamped homogenous electric field on a CHEF-DR III system (Bio-Rad Laboratories) with two liters of standard 0.5X TBE buffer. The gel was run at 6V/cm and reorientation angle at 120° with temperature of 14°C with two block of pulse time ramped from 15.0 seconds to 30.0 seconds for 7 hours and 2.2 seconds to 56 seconds for 15 hours respectively. DNA size standard that was used was PFG lambda ladder (Biolabs, New England) consisting of concatemers ranging from 48.5 to 1,000 kbp. The gel was stained with 0.5 µg/ml ethidium bromide for 30 minutes and destained in distilled water for 25 minutes before viewing under UV transluminator (Ultra-Lum, California).